Glucose protects *E. coli* from death by the *Vibrio cholerae* type VI secretion system

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Abstract

Vibrio cholerae, the causative agent of the intestinal disease cholera, interacts with other bacteria in dense multispecies communities within both host and environmental settings. Using the harpoon-like type VI secretion system (T6SS), *V. cholerae* delivers toxic effector proteins into neighboring cells, causing cell lysis and death. The T6SS is frequently studied in *V. cholerae* using a *qstR** mutant which constitutively expresses the T6SS. A *qstR* V. cholerae* strain can effectively kill target species *Escherichia coli, Aeromonas veronii.,* and T6SS-sensistive *V. cholerae* cells in a standardized lab killing assay, causing a drop in viable cell counts of five orders of magnitude. This study finds that addition of glucose to a standardized killing assay against *qstR* V. cholerae* restores *E. coli* survival by three to four orders of magnitude, though the same effect is not found for *Aeromonas* or T6SS-sensitive *V. cholerae*. A growth assay revealed that *E. coli* doubling time does not affect killing by *V. cholerae.* Additional evidence shows that *E. coli* does not produce a diffusible molecule that represses the T6SS of *V. cholerae*. Investigation by fluorescence microscopy revealed that *E. coli* cells when entirely surrounded by *V. cholerae* cells survive in the presence but not the absence of glucose, which suggests that glucose causes a relevant physiological change in individual *E. coli* cells. We propose that further study should focus on the *E. coli* capsule as a potential mechanism for surviving T6SS attack. This study makes an unprecedented case that attack via the T6SS can be thwarted by sugar metabolism in target cells.

Introduction

Vibrio cholerae, well known as the bacterium responsible for the diarrheal disease cholera, is also an aquatic microbe. In freshwater and marine environments, *V. cholerae* can be found in a planktonic state or attached to surfaces of plants, algae, chitinous zooplankton, and even the guts of fish (Takemura et al 2014). Attached to these surfaces in polymicrobial biofilms, *V. cholerae* can interact, cooperate, and compete with many other bacterial species. One mechanism of bacterial antagonism employed by *V. cholerae* is the Type VI Secretion System (T6SS), a spike that delivers toxic effector proteins into neighboring cells (Pukatzki et al 2007). When an adjacent bacterial cell is punctured by this contact-dependent mechanism, intoxication from these effectors causes cell lysis. This lethal secretion system is found in 25% of gram-negative bacteria (Bingle et al 2008). The T6SS has been hypothesized to serve many purposes, including biofilm defense, biofilm invasion, elimination of non-kin, and killing of phage-infected bacteria (Russell et al 2014). In addition to the direct effects on bacterial cells, the type VI secretion system in *V. cholerae* has recently been implicated in improved intestinal

colonization in mouse and zebrafish models (Zhao et al 2018, Logan et al 2018).

Function of the Type VI Secretion System

The activated type VI secretion system is known to deliver a variety of effector proteins that act on different targets within a cell. The suite of effectors found in a particular strain or species is unique, although similarity between effectors in different species suggests effectors can be acquired through horizontal gene transfer (Thomas et al 2017). Cells prevent self-intoxication from these toxic effector proteins by encoding a cognate immunity protein beside each effector in the genome (Dong et al 2013). In the clinical reference strain C6706 of *V. cholerae*, there are three known effectors with antibacterial properties. TseL is a lipase which breaks down lipids that comprise the cell membrane (Dong et al 2013). VasX is a pore-forming protein which imbeds in the membrane of the target cell, and is implicated in antibacterial as well as anti-eukaryotic activity (Dong et al 2013). VgrG-3, the protein which comprises the "tip" of the type VI secretion system harpoon, has a C-terminal peptidoglycan degrading domain (Brooks et al 2013). Together, these three effectors allow C6706 *V. cholerae* to kill many gram-negative bacterial species including *E. coli*, *Aeromonas veronii*, and even *V. cholerae* cells that are engineered to be deficient in all three cognate immunity proteins. These three species can be used as targets in specialized competition assays called killing assays where *V. cholerae* and its target are co-cultured on solid media, followed by plating on selective media to enumerate target cells that survived a T6SS attack.

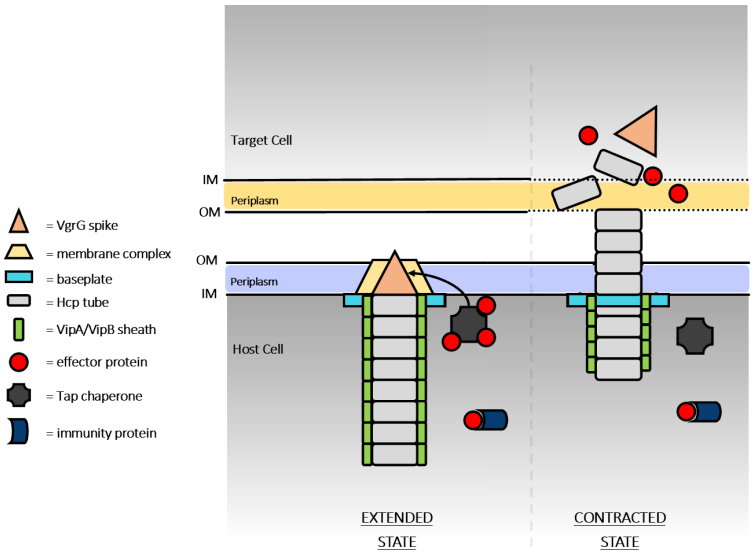


Diagram 1. Schematic of the *Vibrio cholerae* type VI secretion system in its extended and contracted state. Contraction of the T6SS delivers toxic effectors into bacterial target cells, causing cell lysis and death (Crisan unpublished 2018).

Regulation of the Type VI Secretion System

The type VI secretion system in *V. cholerae* clinical strain C6706 is activated by a combination of environmental cues in laboratory settings. When *V. cholerae* is at high cell density, the transcriptional activator HapR responds by up-regulating T6SS and DNA uptake genes (Borgeaud et al 2015). CytR is involved in sensing nucleoside starvation, and up-regulates the T6SS and DNA uptake under starvation conditions, presumably for the purpose of scavenging nucleosides from target cells lysed by the T6SS (Watve et al 2015, Veening and Blockesh 2017). The regulator TfoX is also involved in this pathway; it senses degraded chitin, which is often liberated by *V. cholerae*'s chitinases. In clinical strain C6706, induction of all three

regulators (HapR, CytR, and TfoX) is required for transcription of *qstR*, which is another activator of the T6SS (Watve et al 2015, see Diagram 2). While there are additional regulators of the T6SS including TfoY, OscR, and TsrA, overexpression of *qstR* is sufficient to induce constitutive activity of T6SS in clinical isolates including C6706 (Metzger et al 2017, Ishikawa et al 2012, Zheng et al 2010). *V. cholerae* mutants with a *qstR** mutation are frequently used to study the dynamics of the T6SS in a laboratory environment.

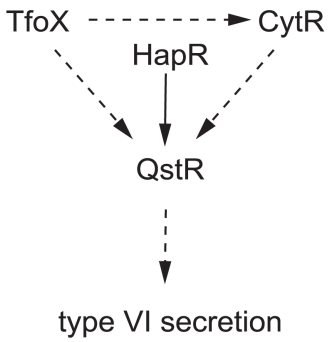


Diagram 2. Proposed regulatory scheme for type VI secretion in *Vibrio cholerae* (Watve et al 2015).

Protection from the Type VI Secretion System

As in many types of biological competition, defense strategies have evolved in response to the lethal type VI secretion system. The facultative pathogen *Pseudomonas aeruginosa* has been shown to sense and respond to type VI attack by assembling its own T6SS machinery and firing back at the competitor (Basler et al 2013). Some species have evolved a more guarded strategy. *Bacillus fragilis* encodes functional immunity proteins that match effectors not encoded in their genome, suggesting a mechanism for resistance against persistent T6SS attack (Wexler et al 2016).

This study explores a new mechanism for survival from the T6SS. Previous work has shown that when co-cultured with *E. coli* in a killing assay, *qstR* V. cholerae* can reduce the survival of *E. coli* by 5 orders of magnitude relative to a T6SS- control (Borgeaud et al 2015). However, the current study shows that upon addition of the monosaccharide glucose to a killing assay, the same *qstR* V. cholerae* only reduced *E. coli* survival by 1 order of magnitude. This drastic increase in survival is shown to be specific to *E. coli* target cells and the monosaccharide glucose. *E. coli*'s survival cannot be explained by differing growth rates or by suppression of the T6SS. Using confocal fluorescence microscopy, *E. coli* was found to survive in the presence of glucose even when physically surrounded by *qstR* V. cholerae* cells. This work provides the foundation for further investigation into the mechanism of survival, which is proposed here to be due to increased production of the *E. coli* capsule in glucose conditions.

Methods

Type VI Secretion System Killing Assay

To measure the strength of the *V. cholerae* T6SS killing phenotype, a killing assay was adapted from previously described methods (Watve et al 2015). *E. coli, V. cholerae*, or *Aeromonas veronii*. cells were grown in overnight at 37˚C in a shaking incubator in liquid Lysogeny Broth (LB) with or without 0.4% glucose. Overnight cultures were diluted to an OD_{600} of 1.0, and then killer and target cells were mixed in a 10:1 ratio. 50 µL of this mixture was plated on Millipore membrane filters (pore size 0.2 µm) placed on LB agar plates with or without 0.4% glucose. Plates were incubated at 37˚C for 3 hours. After incubation, filters were placed in 50 mL falcon tubes and vortexed in 5 mL LB for 30 seconds. The resulting suspension was serially diluted and plated on selective media to enumerate surviving prey cells, which

encoded for resistance to an antibiotic to which the *V. cholerae* killer was sensitive.

Alterations to this standard protocol included changing the target species or the killer strain, using alternative sugars (sucrose, maltose, fructose, lactose, galactose, sucrose), decreasing the concentration of glucose, increasing the killer to target ratio, and including two target species in one killing assay.

Luciferase (lux) Assay

To measure how the gene expression of the T6SS in *V. cholerae* varies in glucose, a transcriptional fusion of a major T6SS promoter to the luciferase operon was used. Cells were grown overnight in LB or LB with glucose. These overnight cultures were diluted 1:100 with or without glucose and allowed to grow for 6 hours before luminescence and OD_{600} measurements were taken to calculate RLU (relative light units).

Growth Assays

Growth assays on solid LB plates with or without 0.4% glucose were conducted to mimic killing assay conditions. *E. coli* and *V. cholerae* cells were grown overnight at 37˚C in a shaking incubator in liquid LB with or without 0.4% glucose. Overnight cultures were diluted to an OD_{600} of 0.1, and then 50 µL of was spotted on Millipore membrane filters (pore size 0.2 µm) placed on LB agar plates without or with 0.4% glucose. The culture on these plates was allowed to dry for 15 minutes, and then an initial CFU measurement was made by removing half of the samples for analysis. These samples were vortexed in 5 mL LB, serially diluted, and plated on LB to count initial colony forming units (CFUs). The remaining samples were placed in an incubator at 37˚C. After 3 hours, the remaining samples were accordingly vortexed, diluted, and plated for CFUs.

Confocal Fluorescence Microscopy

A co-cultured biofilm of *V. cholerae* and *E. coli* was imaged using confocal fluorescence microscopy. Green fluorescent *E. coli* and unlabeled *V. cholerae* were grown overnight at 37˚C in a shaking incubator in liquid LB with or without 0.4% glucose. Overnight cultures were then concentrated to an OD_{600} of 4.0, and killer and target cells were mixed at a ratio of 100 *V. cholerae* to 1 *E. coli*. 1 µL drops were spotted onto agar pads on microscope slides, and then covered with a sterile glass slide. Propidium iodide staining was used to visualize DNA from lysed cells. Seven sets of images were taken during a 5-hour period, and the final images after 5 hours can be seen in Fig. 6.

Results

E. coli resists attack from *V. cholerae* type VI secretion system in presence of glucose.

A killing assay between *V. cholerae qstR** C6706 and *E. coli* target cells was performed in LB with or without glucose. In LB, *E. coli* survival was reduced by five orders of magnitude compared to co-culture with a *V. cholerae* Δ*vasK* non-killer control (Figure 1a). When this assay was completed in glucose conditions, *E. coli* survival against the non-killer control was the same as in LB (Student's t-test, p= 0.486). However, when *E. coli* was co-cultured with *V. cholerae*

*qstR** in glucose, survival was four orders of magnitude higher than in LB (Student's t-test, p<0.0005). This effect was observed even when glucose concentrations were reduced to 0.1% (see Fig 1b). Killing assays were repeated with different sugars including sucrose, fructose, maltose, lactose and galactose (Figure 2). These sugars had modest effects on *E. coli* survival, and none of the sugars produced a phenotype as strong as glucose.

Target cell survival in glucose is killer and target dependent.

To examine the specificity of this enhanced survival on glucose, a variety of killer cells were tested. This included the clinical *V. cholerae* strain V52, whose effectors are identical to C6706 but are regulated differently; four strains of *V. cholerae* originally collected from an environmental setting; and an *Enterobacter* species that kills *E. coli* cells in a T6SS dependent manner. Target *E. coli* cells were killed in both LB with and without glucose by *Enterobacter spp*. and by two of the four environmental strains (See Figure 2a). *E. coli* cells experienced enhanced survival in glucose against V52 and two of the environmental strains.

Additional target cell types were tested against clinical strain C6706 for survival in glucose. *Aeromonas veronii*., a fish gut commensal, was still killed by *V. cholerae* in glucose (Figure 2b). We also tested a T6SS-sensitive, non-killer *V. cholerae* strain engineered with deletions in three immunity proteins and Δ*vasK*. These cells were killed in glucose at levels indistinguishable from LB without glucose.

***E. coli* cells do not "outgrow" *V. cholerae* type VI secretion system mediated killing**

E. coli survival on glucose could be explained if *E. coli* cells are replicating faster than *V. cholerae* cells can kill them. To test this, we used a monoculture growth assay on agar plates that mimicked killing assay conditions. *E. coli* cell counts were not significantly higher in glucose as compared to LB after 3 hours (Student's t-test, p=0.293). In addition, *V. cholerae* cell counts were comparable in both media conditions after 3 hours (Student's t-test, p=0.079).

The *V. cholerae* type VI secretion system is not repressed by *E. coli* or glucose.

A three-way killing assay was performed to determine if the presence of *E. coli* renders *V. cholerae* unable to kill with its T6SS. Target cells included engineered T6SS-sensitive *V. cholerae* cells and *E. coli*. When these cells were co-cultured together with *V. cholerae qstR**, target *E. coli* cells still survived while target *V. cholerae* cells were killed at levels indistinguishable from LB without glucose (Figure 4). Furthermore, a luciferase assay of a transcriptional fusion to a main T6SS promoter revealed that the genes for the T6SS are transcribed in both LB with and without glucose. (Figure 4c).

***E. coli* cells survive on glucose even when surrounded by *V. cholerae* cells.**

Co-culture biofilms on LB agar or LB agar with glucose under a glass slide were visualized on a confocal fluorescent microscope (see Fig. 6). Qualitatively, *E. coli* survival against killer *V. cholerae* in glucose looks similar to survival against nonkiller *V. cholerae*. In LB, killing is evidenced by the drastic reduction in green fluorescent *E. coli*. Replicates and quantification will be conducted in the future to confirm this preliminary result.

Discussion

Vibrio cholerae uses its type VI secretion system to kill neighboring bacterial cells. This has important implications for it survival in both host and environmental settings. *E. coli* is frequently used in laboratory settings as a target for *V. cholerae*'s type VI attack. Here we show that under lab conditions addition of glucose is sufficient to stimulate massive survival of *E. coli* cells against clinical C6706 *V. cholerae* cells that constitutively express the T6SS. While other sugars such as sucrose and fructose also provide modest resistance, glucose has the largest effect on *E. coli* survival. Future research might seek to find a mechanistic explanation for why some sugars lend more resistance than others. It is particularly interesting that maltose failed to produce a large increase in survival, since maltose is a disaccharide composed of two units of glucose. Since K-12 *E. coli* has been shown to metabolize maltose, it is unlikely that this lack of survival is due to a lack of maltose metabolism (Boos et al 1998). There seems to be an important distinction between glucose, which is transported and metabolized as glucose, and maltose, which enters the cell as maltose but gets processed into glucose and α-glucose-1-phosphate inside the cell (Boos et al 1998).

We focused on the dynamics of glucose, since it caused the largest increase in survival out of all the sugars tested. We demonstrate in contrast to clinical isolate C6706, some environmental strains of *V. cholerae* are able to kill *E. coli* in glucose. Clearly, there is some variation amongst *V. cholerae* strains driving this difference, for example the suite of effectors used by different *V. cholerae* strains. Additionally, we show that T6SS+ *Enterobacter spp.* is able to kill *E. coli* in glucose in a contact-dependent manner. This might be an interesting avenue for future research, since killer cell type seems to have an effect on *E. coli* survival on glucose.

We demonstrate that this massive survival of *E. coli* is not simply due to a faster growth rate, since neither *V. cholerae* nor *E. coli* have drastically different cell counts after 3 hours of growth on solid media. To account for the survival found in glucose conditions, *E. coli* cell counts would need to be 3-4 orders of magnitude higher than in LB, but this was simply not the case. A previous study by Borenstein *et al* indicates that *E. coli* cells persist against the *V. cholerae* T6SS when *E. coli* occupies a sufficiently large monoculture domain (2015). In this case, *E. coli* growth on the interior of the domain outpaces T6SS killing, which can only occur at the interface between *V. cholerae* and *E. coli* domains. However, we show that *E. coli* evades killing in well-mixed cultures at ratio of 10:1 and even 100:1 *V. cholerae* to *E. coli*. Individual *E. coli* cells survive even when entirely surrounded by *qstR* V. cholerae.*

V. cholerae cells express their T6SS in glucose conditions as indicated by luciferase reporter assays. They can also assemble, fire, and kill with the T6SS in glucose, as evidenced by their ability to kill *Aeromonas veronii* and immunity-deficient *V. cholerae* cells in glucose conditions. To rule out the effect of *E. coli* on *V. cholerae* T6SS expression, we demonstrate the *V. cholerae* can kill immunity-deficient *V. cholerae* cells even when *E. coli* cells are present. To this end, we have shown that *V. cholerae* regulation of the T6SS is not responsible for survival

of *E. coli* in glucose.

Given that *E. coli* survival cannot be explained by growth of *E. coli* or regulation in *V. cholerae*, the prevailing hypothesis is that glucose induces a physiological change in *E. coli* that confers protection against the T6SS. We propose that *E. coli* survival may be influenced by the production of a capsule. A bacterial capsule is an attached extracellular matrix of polysaccharides. Capsule production is found in a variety of bacterial species including *E. coli*, *Klebsiella pneumonia*, *Haemophilus influenza*, and *Salmonella enterica*. (Gottesman et al 1991, Schouls et al 2008, Yoshida et al 2000, Gibson et al 2006). Commonly recognized as a mechanism of escaping the human immune system, capsules also protect *E. coli* against attack by phage (Scholl et al 2005). Interestingly, the T6SS bears many similarities to a phage tail spike (Pukatzki et al 2007).

E. coli capsule production differs across strains. The strain used in this study, *E. coli* K-12 MG1655, produces colanic acid (Hufnagel et al 2015). Future experiments will focus on testing for the presence of a capsule that allows *E. coli* to withstand type VI secretion system attack by *V. cholerae*. Preliminary tests will focus on visualizing the *E. coli* capsule under different sugar conditions with Anthony's stain. We hope to acquire *E. coli* mutants in the *cps* operon that cannot produce a capsule (Huang et al 2006). We would expect that a *cps-* mutant would be killed by the *V. cholerae* T6SS in glucose, and that a *cps** mutant would survive type VI attack even without glucose. If capsules play a role in evading the type VI secretion system, this would be an exciting opportunity to study how an important strategy of bacteria-host antagonism may also play a role in bacteria-bacteria conflict.

Conclusion

Vibrio cholerae is a deadly human pathogen that lives in both the human body and aquatic settings. It frequently interacts with other microbes, and uses its type VI secretion system to kill bacterial competitors. Here we show that *E. coli*, classically sensitive to the T6SS in lab settings, survives *V. cholerae* type VI attack when glucose is added to the system. We propose that this unexpected survival is due to production of a capsule, a polysaccharide coat typically understood to help pathogens evade the immune system. Understanding how cells resist the *V. cholerae* type VI secretion system can give insight into both the ecology and pathogenesis of this bacterium. *V. cholerae* uses its T6SS to a competitive advantage in host and environmental settings, so this research might help treat and prevent future cases of cholera.

Figures

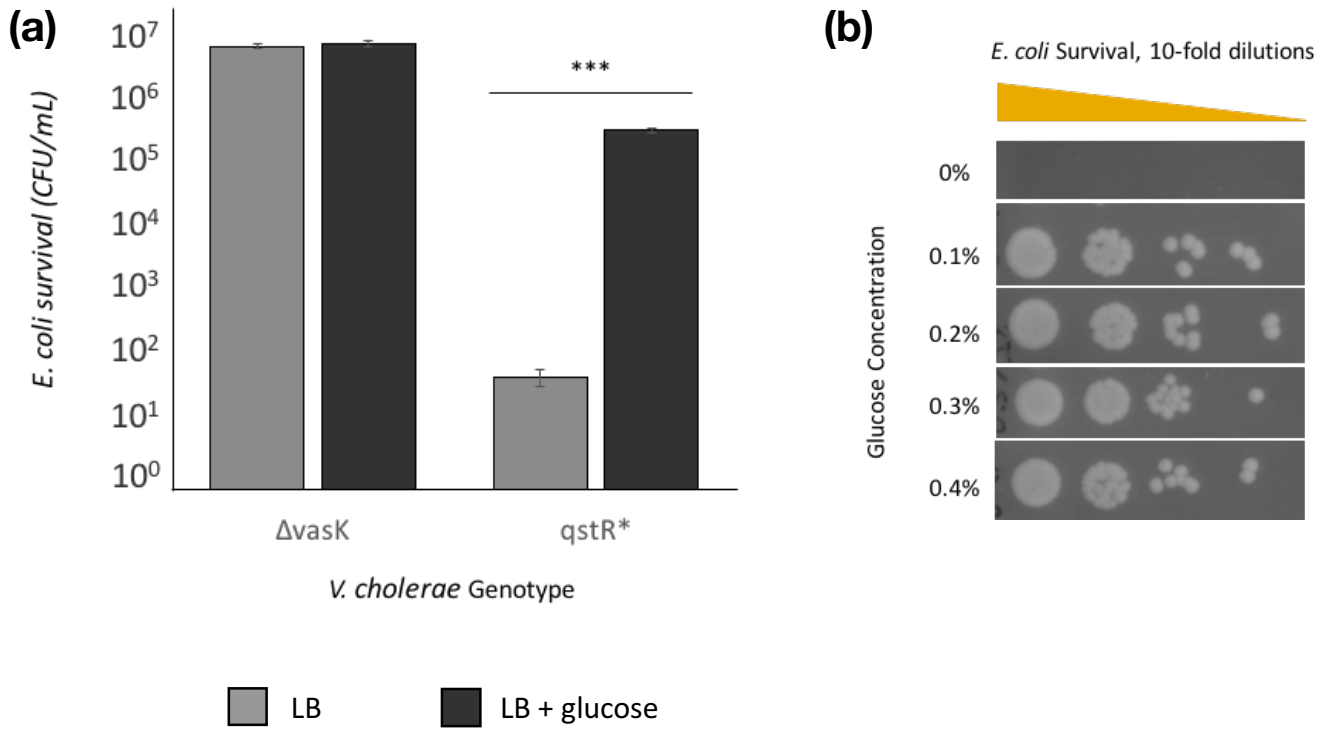
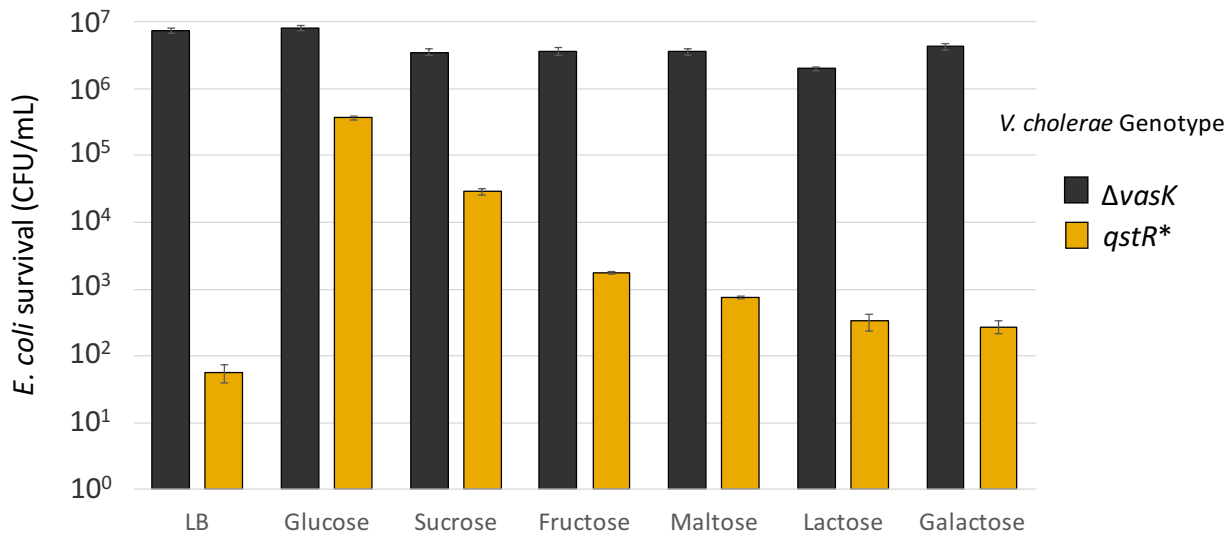


Figure 1. ***E. coli*** **experiences enhanced survival against** ***V. cholerae*** **T6SS on glucose. (a)** Survival of target *E. coli* cells after 3 hours of co-culture with *V. cholerae* cells on LB (grey) or LB supplemented with 0.4% glucose (black) agar. ΔvasK cells are unable to assemble and fire the T6SS while qstR* cells constitutively fire the T6SS. *V. cholerae* cells outnumber *E. coli* cells 10 to 1 at time of innoculation. Assays completed in triplicate (Student's t-test, $p<0.0005$). **(b)** Ten-fold dilutions from left to right showing survival of *E. coli* target cells against *V. cholerae qstR** under different glucose concentrations in a standard killing assay.



<u>Figure 2.</u> Survival of *E. coli* after 3 hours of co-culture with *V. cholerae* cells on LB supplemented with various sugars. *E. coli* cells were co-cultured with T6SS- (black) or T6SS+ (yellow) *V. cholerae* cells for three hours on LB agar supplemented with a sugar. All sugars were added at concentration of 0.4% w/v. Assays completed in triplicate.

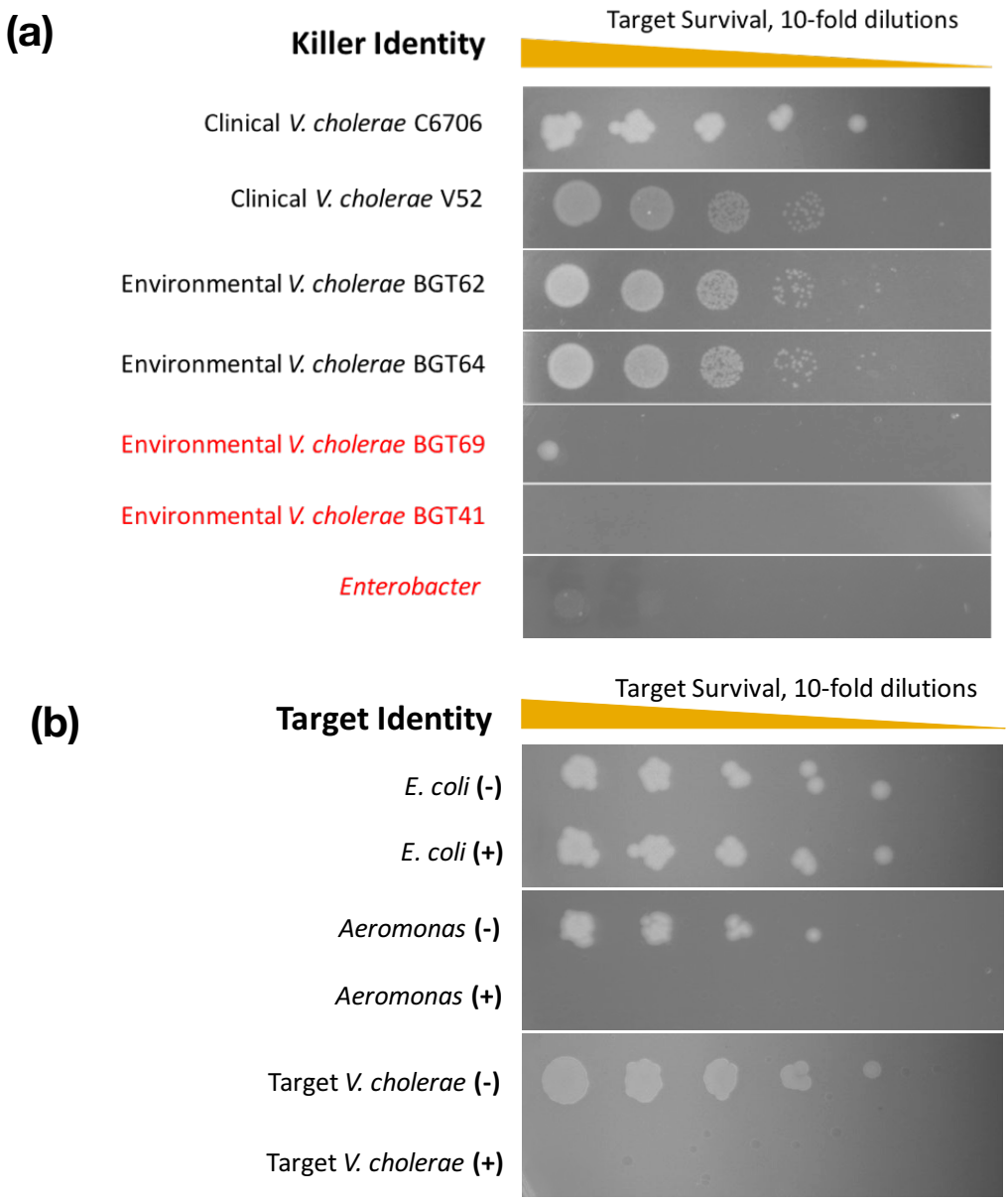
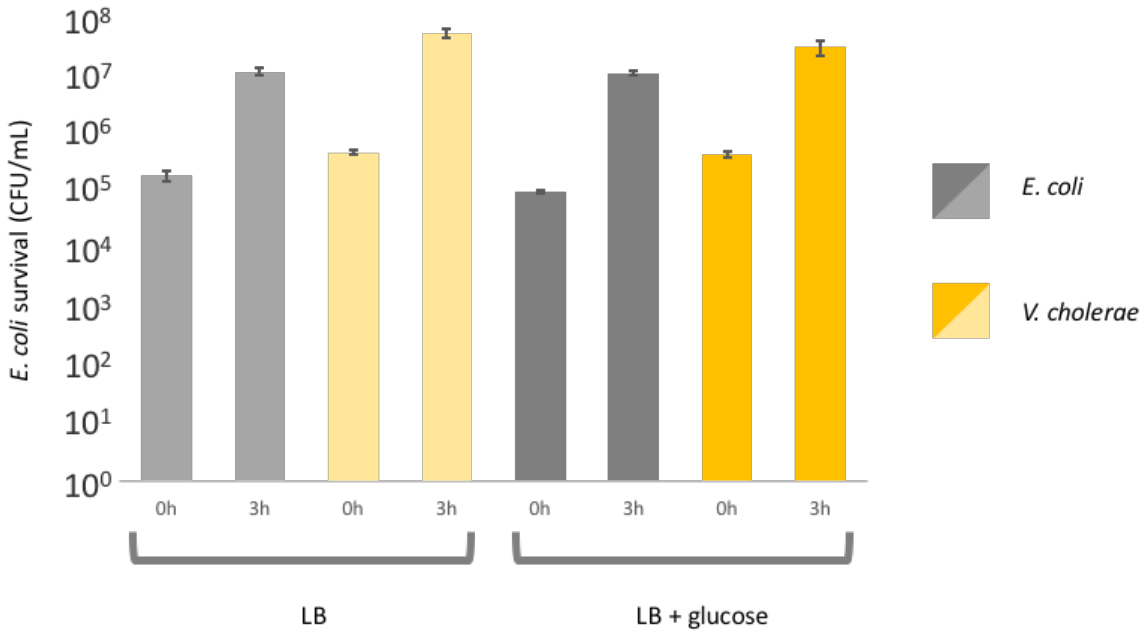


Figure 3. Target survival in glucose is dependent on killer and target cell type. (a) Survival of *E. coli* target cells after 3 hours of co-culture with various T6SS+ killer strains and species on LB agar with 0.4% glucose, shown in ten-fold dilution from left to right. Killer cell types in red are still able to kill *E. coli* in glucose. **(b)** Survival of various target cell types after 3 hours of co-culture with *V. cholerae* on LB agar with 0.4% glucose. Treatments denoted **(-)** represent co-culture with a T6SS- ΔvasK *V. cholerae* mutant while **(+)** represents co-culture with a T6SS+ qstR* *V. cholerae* mutant.



<u>Figure 4.</u> *E. coli* and *V. cholerae* experience similar growth in both LB and LB supplemented with glucose. *E. coli and V. cholerae* cells grown separately on solid agar with or without glucose for 3 hours in triplicate. Growth assay conditions were chosen to mimic conditions of a 3-hour killing assay. This result suggests that *E. coli* survival on LB with glucose is not due to accelerated growth rate. Growth assays completed in triplicate.

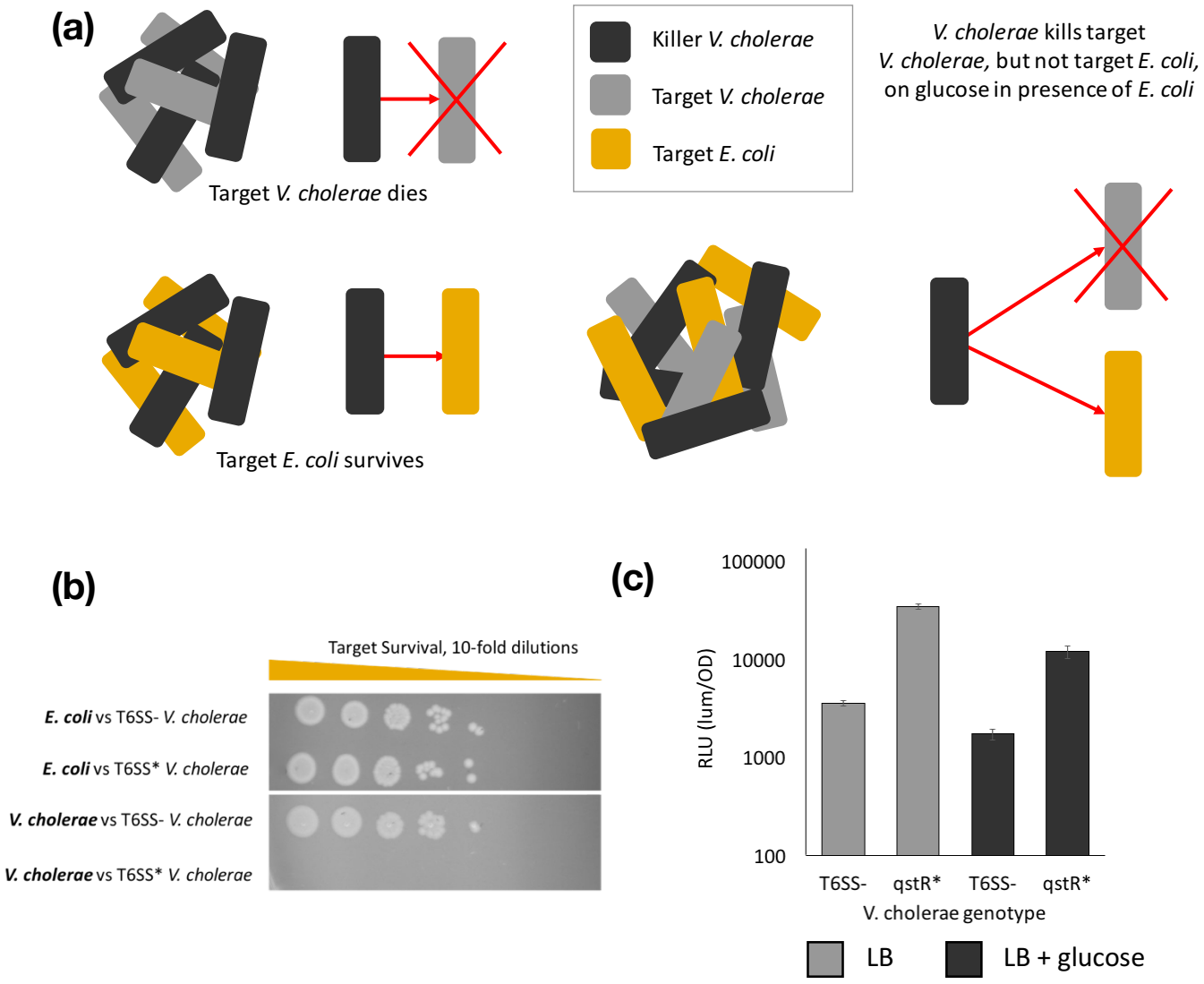


Figure 5. *E. coli* does not repress the *V. cholerae* T6SS. (a) Schematic of three-strain killing assay on LB supplemented with glucose, depicting that *V. cholerae* kills T6SS-sensitive target *V. cholerae* cells even in presence of *E. coli*. **(b)** Survival of target cells (bold) against T6SS* or T6SS- *V. cholerae* cells. On the same agar plate, *V. cholerae* kills *V. cholerae* cells lacking T6SS immunity proteins but *E. coli* cells survive T6SS attack. This result suggests that *E. coli* does not inactivate the T6SS, ruling out the hypothesis that *E. coli* produces a metabolic byproduct or secreted good that prevents T6SS killing. The mechanism of *E. coli* survival is therefore specific to *E. coli* and cannot protect other cell types. **(c)** A luciferase assay reveals activity of a main T6SS promoter in qstR* *V. cholerae* cells even in the presence of glucose.

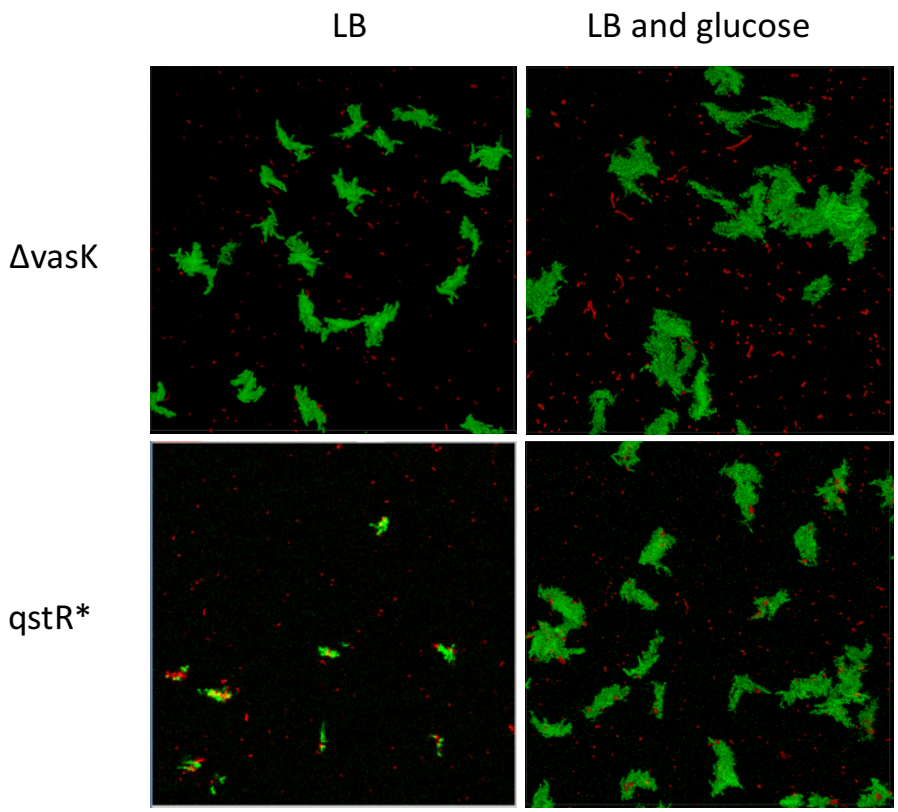


Figure 6. ***E. coli*** **cells survive on glucose even when entirely surrounded by** ***qstR* V. cholerae,*** **suggesting resistance at the single cell level.** Confocal fluorescence microscopy images of a dense biofilm of *E. coli* (green) and *V. cholerae* (unlabeled) under a glass slide after 5 hours of growth on LB agar or LB agar with glucose. Any blank or black space is occupied by *V.* cholerae cells, as visible in the transmission image (data not shown). Red represents dead cells stained with propidium iodide. Cells were inoculated at a ratio of 100:1 *V. cholerae* to *E. coli*. Field of view 152x152 microns.

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